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Research report

The distribution of substance P and neuropeptide Y in four songbird species: a comparison of food-storing and non-storing birds

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Abstract

The distributions of the neuropeptides substance P (SP) and neuropeptide Y (NPY) were investigated in four songbird species that differ in their food-storing behavior. The food-storing black-capped chickadee (*Parus atricapillus*) was compared to the non-storing blue tit (*Parus caeruleus*) and great tit (*Parus major*) within the avian family Paridae, as well as to the non-storing dark-eyed junco (*Junco hyemalis*). All four species showed a similar distribution of SP throughout the brain with the exception of two areas, the hippocampal complex (including hippocampus (Hp) and parahippocampus (APH)) and the Wulst (including the hyperstriatum accessorium (HA)). SP-like immunoreactivity was found in cells of the Hp in juncos, but not in the three parid species. Two areas within the APH and HA showed SP-like immunoreactivity in all four species. The more medial of these (designated SPm) is a distinctive field of fibers and terminals found throughout the APH and extending into the HA. A positive relationship between SPm and Hp volume was found for all four species with the chickadee having a significantly larger SPm area relative to telencephalon than the other species. The distribution of SP in this region may be related to differences in food-storing behavior. In contrast to substance P, NPY distribution throughout the brain was similar in all four species. Further, NPY-immunoreactive cells were found in the Hp of all four species and no species differences in the number of NPY cells was observed.

Theme: Neural basis of behaviour

Topic: Neuropeptides and behaviour

Keywords: Substance P; Neuropeptide Y; Hippocampus; Wulst; Spatial memory; Food storing

1. Introduction

The Wulst and hippocampal complex (HC) show substance P (SP) and neuropeptide Y (NPY)-like immunoreactivity in pigeons [4,14,49] and quail [3]. However, no one has described in detail the immunoreactivity of these neuropeptides within the songbird brain or quantified the distribution of these neuropeptides among songbird species. Examining the distribution of these peptides in song birds is interesting because SP and NPY are implicated in memory processes in other vertebrates (SP [27,35,55]; NPY [17,18]) and some species of song birds are known to use the hippocampus in forming spatial memories of the locations of stored food items [51]. Our hypothesis was that the expression or distribution of SP and NPY might differ in the hippocampus and related structures between species with known differences in spatial memory for stored food.

Abbreviations: APH, parahippocampal area; CoS, nucleus commissuralis septi; DLM, dorsolateral anterior thalamic nucleus, pars medialis; DMP, dorsomedial posterior thalamic nucleus; DVR, dorsal ventricular ridge; FDB, nucleus of the diagonal band; FPM, medial forebrain bundle; HA, hyperstriatum accessorium; HC, hippocampal complex; HD, hyperstriatum dorsale; HIS, hypertriatum intercalates superior; Hp, hippocampal complex; HVC, higher vocal center; IHA, intercalates hyperstriatum accessorium; LPO, lobus parolfactorius; MAN, magnocellular nucleus of the anterior neostriatum; N, neostriatum nSt, nucleus striaterminalis; NPY, neuropeptide Y; PA, paleostriatum augmentatum; PP, paleostriatum primativum; SC, dnucleus subcoeruleus, pars dorsalis; SL, lateral septum; SLd, dorsal lateral septum; SLv, ventral lateral septum; SM, medial septum; SP, substance P; SPf, substance P field; SPl, lateral substance P field; SPm, medial substance P field; T, telencephalon; Tn, nucleus taeniae; TP, nucleus tegmenti pedunculo-pontinus

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The avian hippocampus is homologous to the mammalian hippocampus based on development [29,52], connectivity [10,54], and distribution of neurotransmitters [14,15,34]. Like the hippocampus in mammals, it is also important in spatial memory. Lesions to the avian hippocampus disrupt memory for food storage sites [46], homing ability [8,19], and performance on many tests of spatial memory [12]. Food storing birds also have a larger relative hippocampus than non-storers within many avian families [6,24–26,33,45], which is associated with their superior performance on tasks of spatial memory (reviewed in Ref. [47]).

The importance of the avian hippocampus in spatial memory behavior has been established. Much less is known about which brain structures besides the hippocampus are involved in forming or using spatial memories. Our study looks for differences between food-storing and non-storing bird species in the size or cell numbers of other brain areas as indicators of possible involvement in this behavior. Particular attention is paid to a zone termed the substance P field (SPf) and the Wulst because of previous research on their function or connectivity. The SPf is comprised of a dense complex of fibers and terminals that are immunoreactive for substance P (SP-ir). It is located in a region adjacent to the hippocampus (Hp) [14,49,53]. We also find SP-ir lateral to the SPf (below) and so will designate the SPf as the medial substance P field (SPm). The SPm forms the lateral boundary of the hippocampus (Fig. 1) and comprises much of the parahippocampal area (APH). The SPm has been suggested to be the avian counterpart to portions of the mammalian entorhinal cortex based its placement, connections, and immunoreactivity [14,53]. In mammals, the entorhinal cortex is important in spatial memory [28,39].

Another region of SP-ir is found within the hyperstriatum accessorium (HA). We designate this area the lateral substance P field (SPl) (Fig. 1). Subfields in the mid and caudal Wulst (which includes HA and SPl) are thought to be the primary visual area in birds. The avian Wulst carries out many of the same functions as the mammalian neocortex [30,37]. Visual information from the primary visual and visual association cortices in mammals is sent to the hippocampus via connections in the entorhinal cortex. Parts of the Wulst may play a similar role in birds. Indeed, it has been suggested that the Wulst may be important in acquisition of familiar landmark navigation by homing pigeons [7].

The present study examines the distribution of substance P (SP) and neuropeptide Y (NPY) across the brains of four wild-caught species that differ in food-storing and retrieving. We study three species of birds in the parid family: the North American black-capped chickadee (*Parus atricapillus*), which stores food, and the European great tit (*Parus major*) and blue tit (*Parus caeruleus*), which do not store food. We also look at the North American dark-eyed junco (*Junco hyemalis*), a species outside the parid family that does not store food but lives in a similar habitat with a similar diet as parids. In these four species we describe the general distribution of SP- and NPY-like immunoreactivity throughout the forebrain. We also describe in detail the distribution of SP- and NPY-like immunoreactivity within the Hp, APH, and HA and measure the volume of the two distinct SP-ir areas, SPm and SPl, within the APH/HA region.

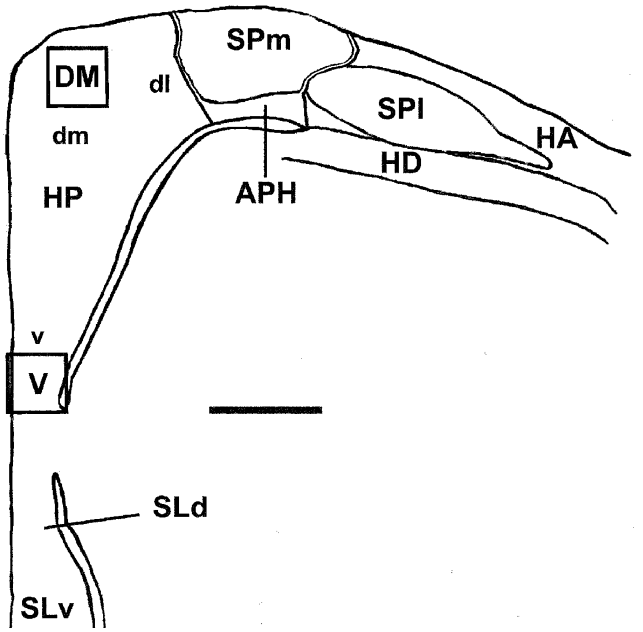

Fig. 1. A diagram of a coronal section of the avian hippocampus, parahippocampus, Wulst, and septum showing structures in the right hemisphere. The squares are the location of the grid used for counting labeled NPY+ cells in the hippocampus. The hatched line lateral to the Hp indicates the boundary of the parahippocampus. APH, parahippocampus; HA, hyperstriatum accessorium; HD, hyperstriatum dorsale; HP, hippocampus; V, ventral area of the hippocampus included in NPY cell count; v, ventral hippocampus; dl, dorsolateral hippocampus; DM, dorsomedial area of the hippocampus included in NPY cell count; dm, dorsomedial hippocampus; SLd, dorsal lateral septum; SLv, ventral lateral septum; SPm, medial SP field; SPl, lateral SP field. Scale bar=500 μm.

2. Materials and methods

2.1. Animals

Eight black-capped chickadees (four males/four females) and four dark-eyed juncos (two males/two females) were captured in the Ithaca, NY, area between March and April 1998. Eight great tits (four males/four females) and seven blue tits (three males/four females) were captured in Brecht, Belgium, during March 1998.

2.2. Preparation of the brains

All birds were anesthetized (Chloropent, Fort Dodge Labs) and transcardially perfused with 0.9% saline and

0.1% sodium nitrite in a 0.1 M sodium phosphate buffer (PB) solution (pH 7.4), followed by 4% paraformaldehyde in PB. Adequate measures were taken to minimize pain or discomfort. The brains were removed immediately and kept at 4°C until sectioning. They were placed in 30% sucrose–4% paraformaldehyde–PB until they sank and then embedded in 10% gelatin–30% sucrose. The gelatin blocks were hardened in 4% paraformaldehyde–PB, then frozen and sliced coronally at 40 μm. Sections were collected in cryoprotectant [58] and stored at −20°C until processed for immunohistochemistry. For each antigen studied, a series of every sixth coronal section was immunolabeled. An adjacent series of sections was stained with cresyl violet to determine boundaries of the hippocampus in order to calculate overall hippocampal and telencephalic volumes. The brains of two male black-capped chickadees and one male blue tit were not well immunolabeled for SP, so were not used in the analysis of that neuropeptide.

2.3. Immunohistochemistry: substance P

Unless otherwise noted, all steps in the immunohistochemical procedure were carried out at room temperature. Sections were removed from cryoprotectant and washed 2×10 min in 0.1 M PB, then in three, 5-min washes with increasingly dilute buffer (0.1 M PB, 0.05 M and 0.025 M PB). They were treated for 10 min in 0.3% H_2O_2 to inactivate endogenous peroxidases, followed by three 5-min washes in 0.025, 0.05 and 0.1 M PB, respectively. The sections were incubated first in 1.5% normal rabbit serum (NRS) added to 0.3% Triton X-100 (Sigma, St. Louis, MO) in PB (PBX) for 30 min, then in monoclonal rat anti-substance P antiserum (1:1000; PharMingen, San Diego, CA) in 1.5% NRS–PBX solution for 72 h at 4°C, and finally for 1 h in biotinylated rabbit anti-rat IgG (1:200; Vector Labs, Burlingame, CA) in PBX. After 1 h in ABC solution (1:50; Vector Labs, Burlingame CA), sections were exposed to 3,3′-diaminobenzidine tetrahydrocholoride (DAB) for 15 min and then to DAB with H_2O_2 (Sigma Fast DAB tablet set, Sigma) and 0.25% $NiCl_2$ for an additional 15 min. Sections were rinsed in PB after each of the preceding incubations. The immunoreacted sections were mounted onto gelatin-coated slides, dried, dehydrated and coverslipped with Permount. To rule out non-specific labeling that would result from the secondary antibody binding to unknown molecules, we carried out the same immunohistochemical protocol omitting the primary antiserum.

2.4. Immunohistochemistry: neuropeptide Y

Unless otherwise noted, all steps in this immunohistochemical procedure were carried out at room temperature and sections were washed initially and between each incubation. The sections were incubated in 1.5% normal goat serum (NGS) in 0.3% PBX for 3 h, then in rabbit anti-neuropeptide Y antiserum (1:10 000; DiaSorin, Stillwater, MN) in PBX solution for 18 h at 4°C, followed by 90 min in biotinylated goat anti-rabbit IgG (1:200; Vector Labs, Burlingame, CA) in PBX, then 2 h in ABC solution (1:50; Vector Labs, Burlingame CA) in PB, and finally in DAB with H_2O_2 (Sigma Fast DAB tablet set, Sigma) and 0.25% $NiCl_2$ for 6 min. The sections were mounted onto gelatin-coated slides, dried, dehydrated and coverslipped with Permount. To rule out non-specific labeling that would result from the secondary antibody binding to unknown molecules, we carried out the same immunohistochemical protocol omitting the primary antiserum.

2.5. Analysis: volume measures

The overall volume of the hippocampal region (Hp) and overall volume of the entire telencephalon were measured using the cresyl violet stained sections. Boundaries for Hp were determined in accordance with published cytoarchitecture criteria for a variety of songbird species [33,45]. All sections on each slide were captured with a video camera (COHU) and digitized on a Macintosh IIci using NIH Image 1.54. The surface area of each HP or telencephalon was outlined and measured. Volumes were calculated by multiplying the surface area by the distance between the center planes of the measured sections (every sixth section=240 μm). Data collection was done blind to the species being measured.

2.6. Analysis: substance P

The general distribution of substance P-like immunoreactivity (SP-ir) throughout the entire forebrain and mesencephalon was recorded, with special attention to the labeled neuropil, cells, fibers and terminals in the Hp, APH, and HA. The substance P-containing field (SPf) in APH/HA described in the pigeon [14,49] and the zebra finch [53] was seen in all four species studied here as the more medial of two histologically distinct, adjacent but separate SP-ir fields. The overall volumes of these two areas, SPm and SPl, were calculated in the same manner as overall hippocampal and telencephalic volumes, except that the video camera captured camera lucida tracings of these areas. Both tracing and measuring were done without knowledge of the species being measured.

2.7. Analysis: neuropeptide Y

The general distribution of neuropeptide Y-like immunoreactivity (NPY-ir) throughout the entire forebrain and mesencephalon was recorded with special attention to labeled cells, fibers, and terminals in the Hp. The number of NPY-ir cells within the ventral (V) and dorsomedial (DM) Hp was calculated by counting NPY-ir cells in three sample sections of the left Hp in each brain. V and DM Hp

were chosen because these two areas show anatomical differences in connectivity and neuropeptide distribution [10,14,34,53,54]. The three sample sections were located 1/4, 1/2, and 3/4 of the way through the rostrocaudal extent of the Hp. Within each of these sections, cells were counted in a 0.08-mm^2 area for both V and DM (illustrated in Fig. 1). All NPY stained cells that were within the area by more than 50% of the total cell body were counted and recorded. The number of cells from the three sections was combined to get a sample cell count for V and for DM. All counting was done without knowledge of the species being measured.

3. Results

3.1. Distribution of substance P cells and fibers outside the hippocampal complex and Wulst

3.1.1. Telencephalon

SP-ir was seen in the hippocampal complex (HC) including HP and APH, Wulst, dorsal ventricular ridge (DVR), and dorsal and lateral pallial areas. Only the DVR (which includes the ectostriatum, neostriatum and ventral hyperstriatum) and the dorsal and lateral pallial areas are discussed in the following paragraph. The HC and Wulst will be described in detail in a separate section.

In the DVR, substance P positive (SP+) fiber staining was seen in the nucleus basalis and ectostriatum. We also found SP-ir in nucleus teniae (Tn), where light SP+ fiber staining was found rostrally and darker SP+ fibers were observed caudally. Many song system nuclei also showed SP-ir. The high vocal center (HVC) and magnocellular nucleus of the anterior neostriatum (MAN) showed light SP+ fibers. Fibers were also seen in the general region of Field L and nucleus interfacialis (NIf) (both in neostriatum (N) ventral to HVC). The fibers in Tn were continuous with the fibers in both NIf and HVC and encompassed a broad area that surrounded these two song system nuclei as well. SP-ir was not observed in the caudolateral neostriatum. In the dorsal and lateral pallidum, SP+ fibers extended from the Wulst to the temporoparieto-occipital region (TPO).

In the basal telencephalon, SP-ir was found in the basal ganglia (lobus parolfactorius (LPO), paleostriatum primativum (PP), paleostriatum augmentatum (PA), olfactory tubercle, ventral pallidum and nucleus accumbens), nucleus stria terminalis (nST), medial forebrain bundle (FPM), and the septum. SP-ir in the basal ganglia and FPM was similar to that described in detail for pigeons [2,36,42] and staining in the basal ganglia is depicted here for the purpose of comparison with the pigeon (Fig. 2). In our four species of song birds, we also observed SP+ fibers in area X, a nucleus within the LPO that is part of the avian song system. These fibers, which have not previously been described, appeared continuous with the dorsal fiber staining in the rest of LPO rostrally, but were contained solely within Area X more caudally.

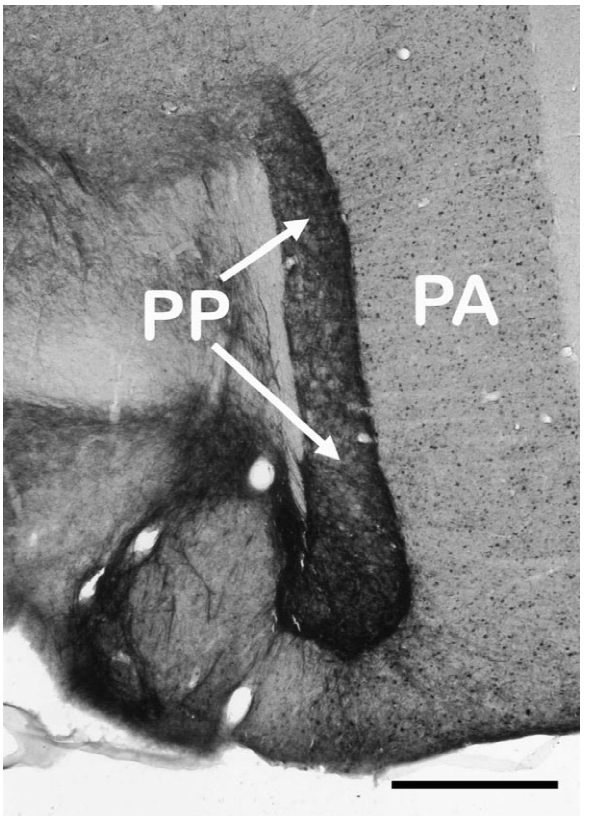

Fig. 2. A photomicrograph of a coronal section in the right hemisphere of the black-capped chickadee showing substance P immunoreactivity in the basal ganglia. PA, paleostriatum augmentatum; PP, paleostriatum primativum. Scale bar=500 μm.

Within the septal region, densely stained fascicles of fibers and sparse cells were located in the medial septum (SM). In the rostral ventral lateral septum (SLv), a few cells scattered among dark SP+ bundles of fibers were seen. In the caudal septum, SP+ fibers were found throughout the SLv, along with SP+ terminals surrounding negatively stained cells (Fig. 3). Along the border between SLv and the dorsal lateral septum (SLd), there was a band of fine SP+ fibers. The nucleus of the diagonal band (FDB) contained densely stained fibers, as did the nucleus of the commissural septi (CoS). The CoS also had a few SP+ cells.

3.1.2. Diencephalon and mesencephalon

In the hypothalamus and thalamus, the distribution of SP+ elements was similar to that previously described in the pigeon [4,20,23,42,56]. Two nuclei that are part of the motor pathway for song production, the dorsomedial posterior thalamic nucleus (DMP) and the dorsolateral anterior thalamic nucleus, pars medialis (DLM), contained SP+ fibers. There was also very light SP+ labeling of fibers in the auditory nucleus ovoidalis and its tract, which is part of the auditory pathway in songbirds.

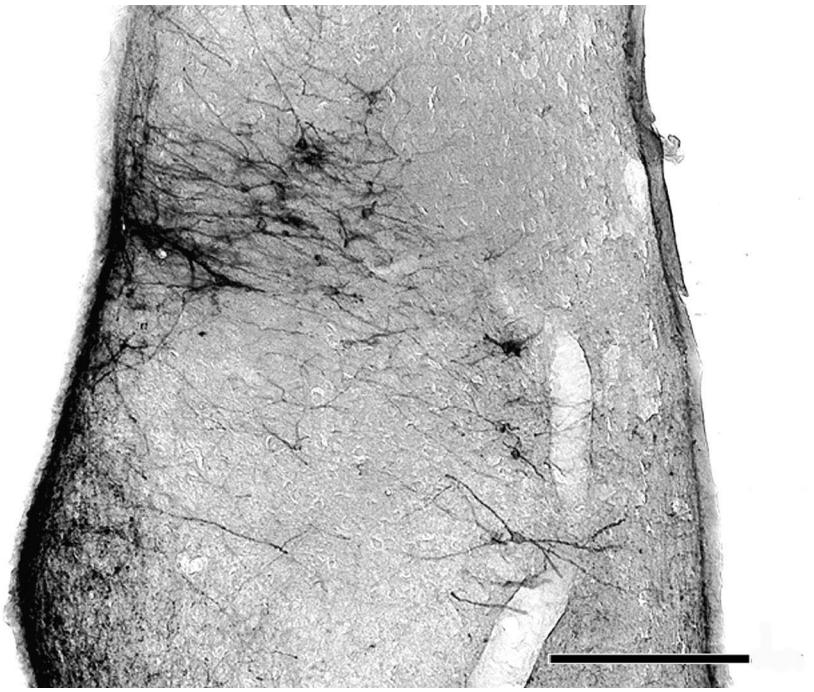

Fig. 3. A photomicrograph of a coronal section in the left hemisphere of the black-capped chickadee showing substance P immunoreactivity in the caudal lateral septum. Scale bar=200 μm.

Throughout the optic tectum, SP+ fibers were sparsely distributed in a laminar pattern similar to that previously described [4,5,13]. SP+ fibers were also found in the nucleus intercollicularis restricted to its dorsomedial nucleus, a part of the motor pathway for song production. This labeling has been described in two additional species of songbirds [5].

In the midbrain outside the tectum, SP-ir was similar to that previously described in pigeons [2,20,42]. This included a well-defined SP-ir tract that is depicted here (Fig. 4) because it connects telencephalon to both diencephalon and mesencephalon and because it serves as a basis for comparison with that previously shown in pigeons [42]. Thick, dark SP+ fibers in the FPM (Fig. 4A) passed through the stratum cellulare externum rostrally (Fig. 4B) and into the ventral tegmental area and the nucleus tegmenti pedunculo-pontinus (TP) more caudally (Fig. 4C,D). The TP is considered the avian equivalent of the mammalian substantia nigra [42]. Part of this fiber tract continued caudally, terminating in the nucleus subcoeruleus, pars dorsalis (SCd) (Fig. 4E). This SP-ir tract descending from the LPO to the TP and SCd has been previously described in pigeons [42] and is similar to mammalian connections between the striatum and substantia nigra.

3.2. Distribution of substance P cells, fibers, and terminals in the HC and Wulst

The distribution of SP-like immunoreactivity in the HC and in the HA and HD areas of the Wulst can be seen in Fig. 5. This figure illustrates staining in the Great Tit, but is similar in all four species. Very dense SP-ir was evident throughout the ventral HA along its boundary with HD and in the dorsal parahippocampus (APH) at the lateral boundary of Hp throughout its rostral-caudal extent. We designated the medial area within the APH as SPm and the lateral area within the HA as SPl (Figs. 1 and 5). Both areas were comprised of densely stained terminals and fibers. The immunolabeling of SP+ fibers and terminals has been noted in the area we call SPm in previous studies [4,14,49].

SPm and SPl first appeared at the same rostral level in the telencephalon (Fig. 5A). They moved laterally as they extended caudally and reached their maximum area at the middle portion of the HC. SPl included both dorsolateral and ventral HA and included an area of intense staining along the HD border (Fig. 5B). Caudal to this level, SPm included an area of more intense staining in its dorsal region, while SPl extended more laterally (Fig. 5C). SPm extended further caudally than SPl (Fig. 5D) and ended near the caudal portion of the HC (Fig. 5E). This pattern of staining was laminar in appearance with darker staining in the most dorsal ‘layer’ of SPm (Fig. 5B–D) and the ventral ‘layer’ of SPl (Fig. 5B).

In the three tit species (black-capped chickadee, great tit, blue tit), very sparse SP+ fibers were observed throughout the Hp and no SP+ cells. However, in the junco, almost all of the Hp contained beaded fibers. Rostral Hp in the junco also had a few scattered SP+ cells along the ventricle (Fig. 6A). More caudally in the junco, the cell

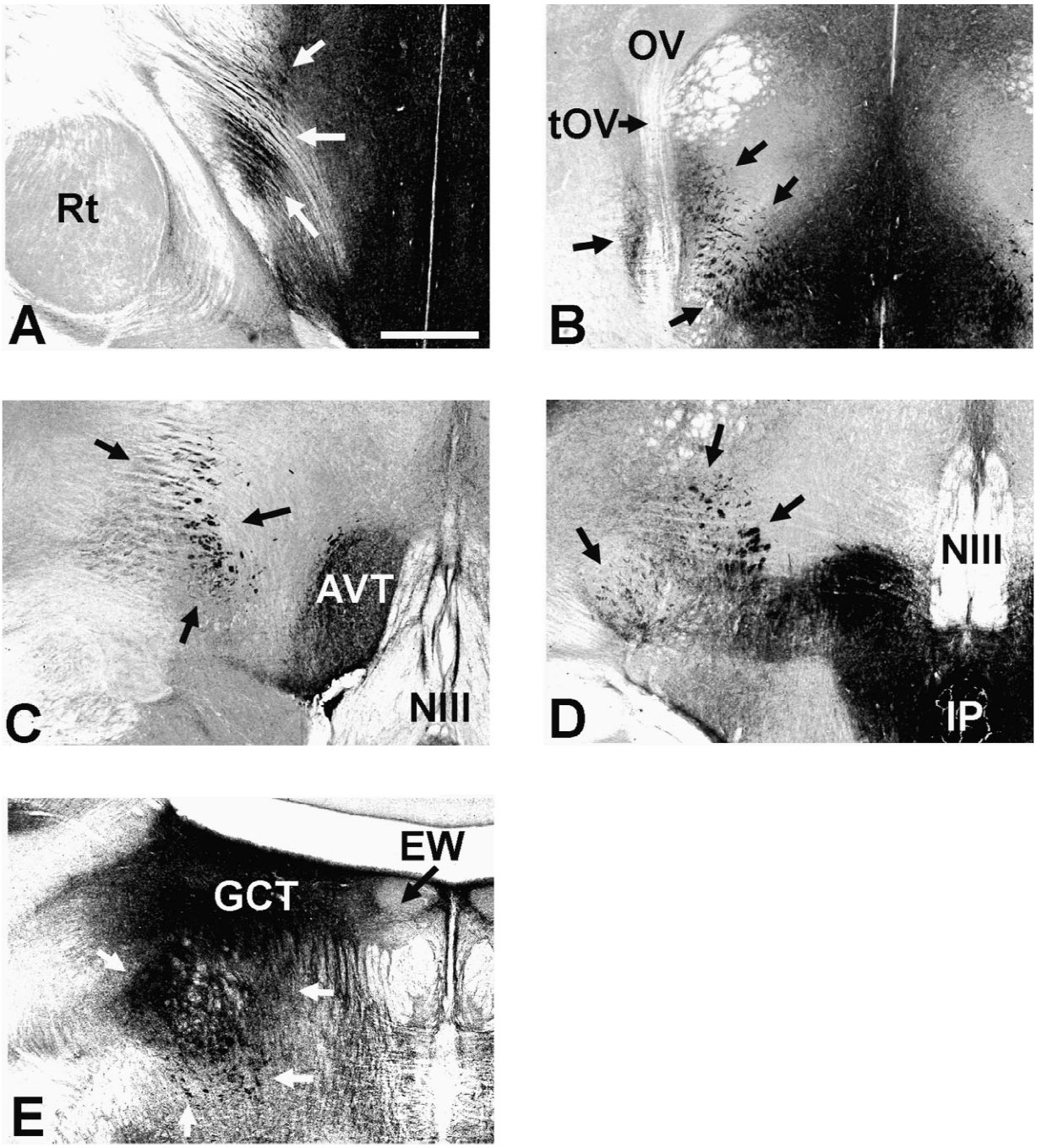

Fig. 4. A series of photomicrographs of coronal sections in the left hemisphere of the blue tit showing a substance P-containing fiber system. Scale bar in A=500 μm and is the same for all photos (A–E). (A) Arrows pointing to substance P+ fibers in the FPM. Rt, Nucleus rotundus. (B) Arrows pointing to substance P+ fibers in the stratum cellulare externum. OV, nucleus ovoidalis; tOV, nucleus ovoidalis tract. (C) Arrows pointing to substance P+ fibers in the nucleus tegmenti pedunculo-pontinus. Substance P+ fibers were also found in the ventral tegmental area (AVT). NIII, oculomotor nerve. (D) Arrows pointing to substance P+ fibers in the tegmenti pedunculo-pontinus. IP, nucleus interpeduncularis. (E) White arrows pointing to substance P+ fibers in the nucleus subcoeruleus, pars dorsalis. Substance P+ fibers were also found in the substantia grisea centralis (GCT). EW, nucleus of Edinger-Westphal.

density increased (Fig. 6B) and immunolabelled cells were found more medially in the Hp (Fig. 6C). The cells occurred throughout the remaining caudal extent of Hp, continuing beyond the caudal extent of SPm (Fig. 6D). The distribution and abundance of SP+ cells and fibers in the junco Hp (Fig. 7) were similar to those described in pigeons [4,14,49].

3.3. Quantitative interspecies comparisons of substance P

The general linear model (GLM) analysis of covariance was used to test for species differences in volumes of the hippocampus and the two SP areas relative to telencephalon. There was a significant ($P<0.05$) species differ-

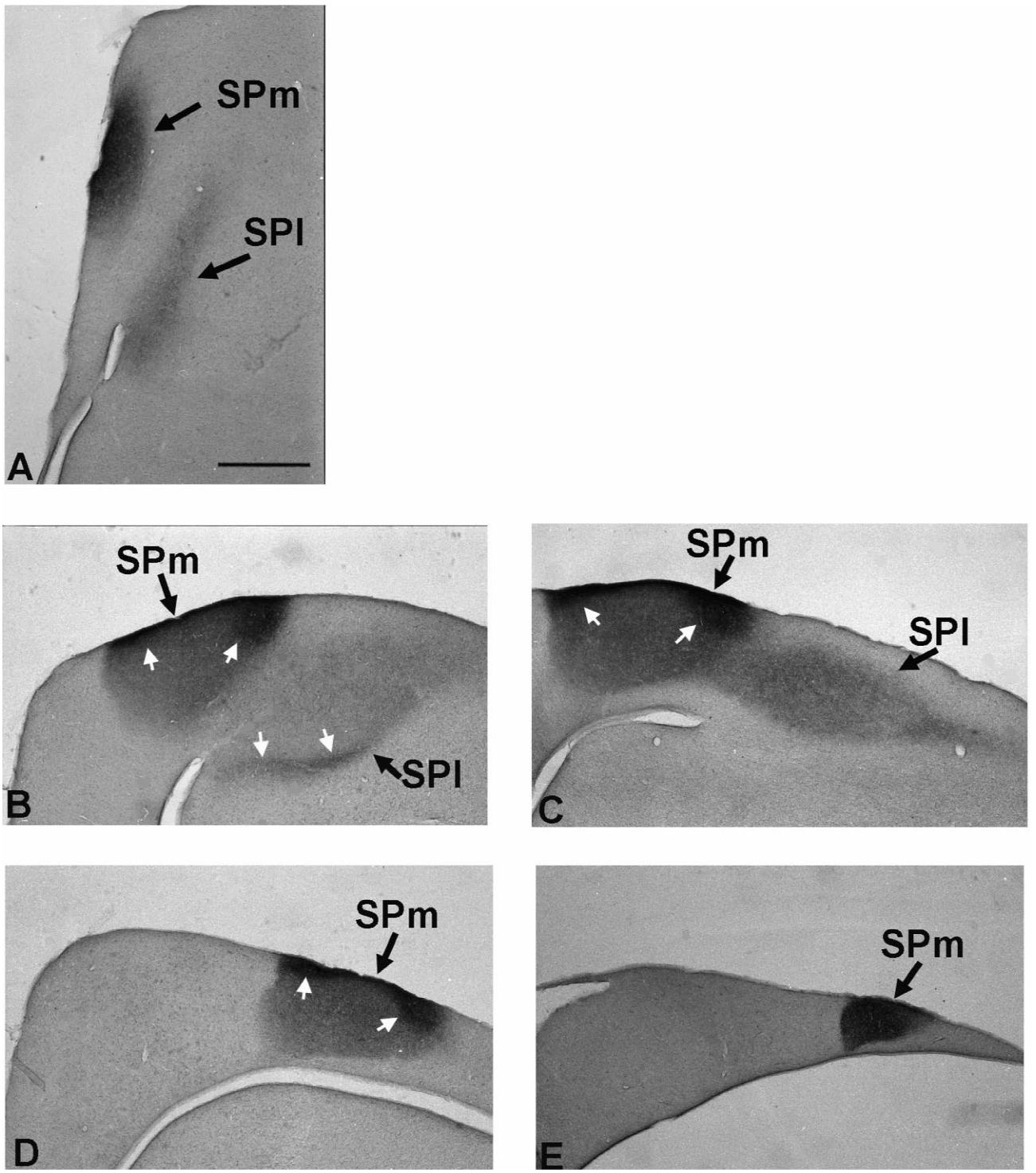

Fig. 5. (A–E) A series of photomicrographs of coronal sections in the right hemisphere of the great tit showing the location of the two substance P areas, SPm and SPl, in the parahippocampus and Wulst. These locations were common to all four species of birds in this study. White arrows are depicting areas of darker immunoreactivity within each area. Scale bar in A=500 μm and is the same for all photos (A–E).

ence in relative Hp volume ($F(3,23)=13.58$, $P<0.001$). A Fisher's LSD post-hoc test revealed that black-capped chickadees had a significantly larger relative Hp volume than juncos, great tits, and blue tits (Fig. 8A). There was also a species difference in relative SPm volume ($F(3,23)=3.69$, $P=0.03$). A Fisher's LSD post-hoc test showed that black-capped chickadees have a larger SPm area relative to T than juncos, great tits, and blue tits (Fig. 8B). There were no species differences in relative SPl volume ($F(3,23)=2.58$, $P=0.083$) (Fig. 8C).

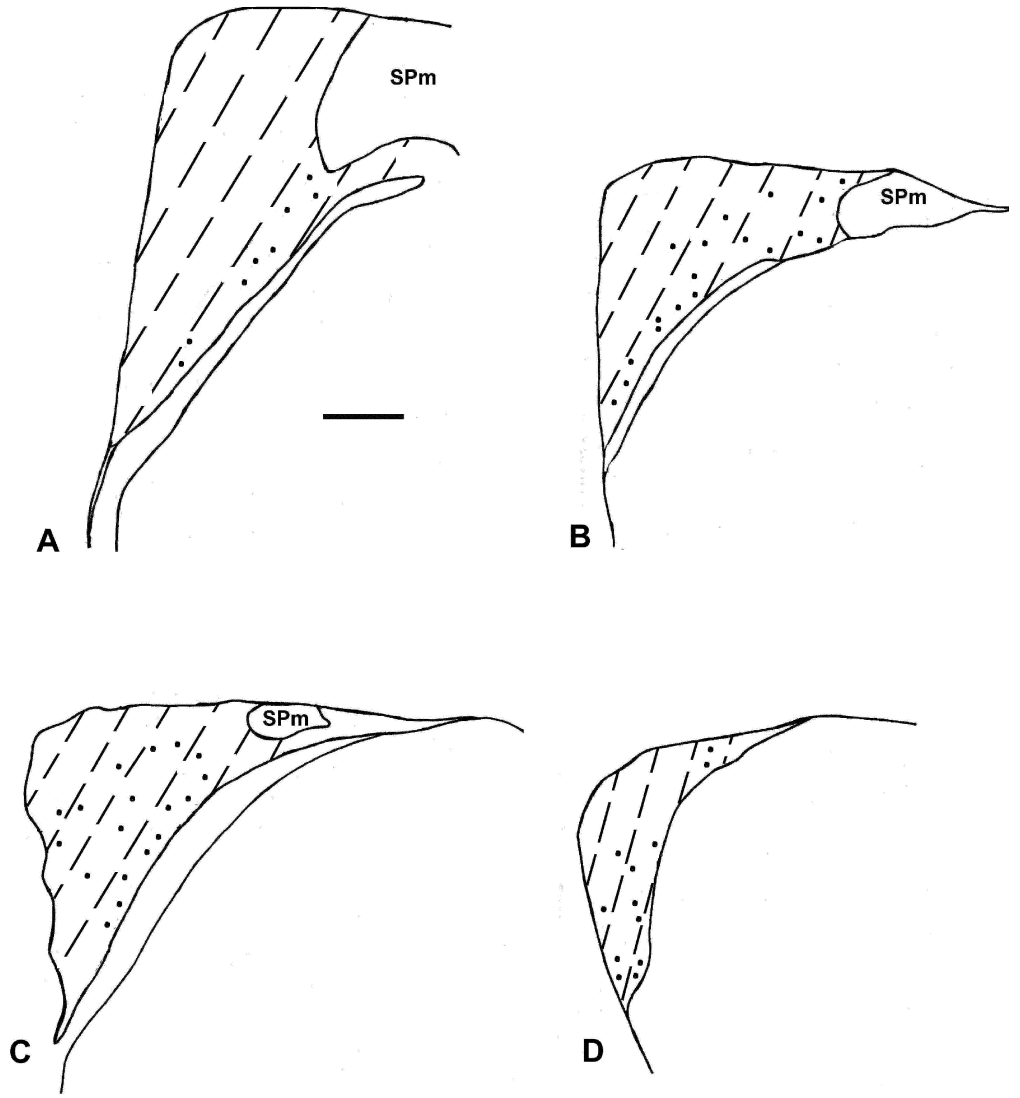

Fig. 6. (A–D) A series of diagrams of coronal sections in the right hemisphere showing the location of substance P+ cells and fibers in the hippocampus of the dark-eyed junco. Dots represent cells and dashed hatching represents fiber staining. Scale bar=500 μm.

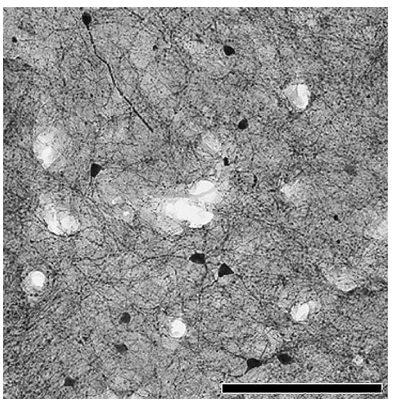

Fig. 7. A photomicrograph of a coronal section in the right hemisphere showing substance P+ cells and fibers in the hippocampus of the dark-eyed junco. Scale bar=200 μm.

Linear regression using the volumes of the telencephalon (T), hippocampus (Hp), and the two SP areas, SPm and SPl across all animals revealed a significant positive correlation between SPm and Hp (r^2=0.33, $F(1,22)$=13.64, $P<0.005$) (Fig. 9A) and between SPm and T (r^2=0.23, $F(1,22)$=6.70 $P<0.02$) (Fig. 9B).

Distribution of neuropeptide Y cells and fibers outside the hippocampal complex

3.4. Telencephalon

NPY-ir was seen in the HC, Wulst, DVR, and dorsal and lateral pallial areas of the telencephalon. The hippocampal complex (HC) will be discussed in detail in a later section. The Wulst, the dorsal ventricular ridge (DVR) and the dorsal and lateral pallium showed similar NPY-ir in all four species.

In the rostral Wulst, NPY+ cells and fibers were found

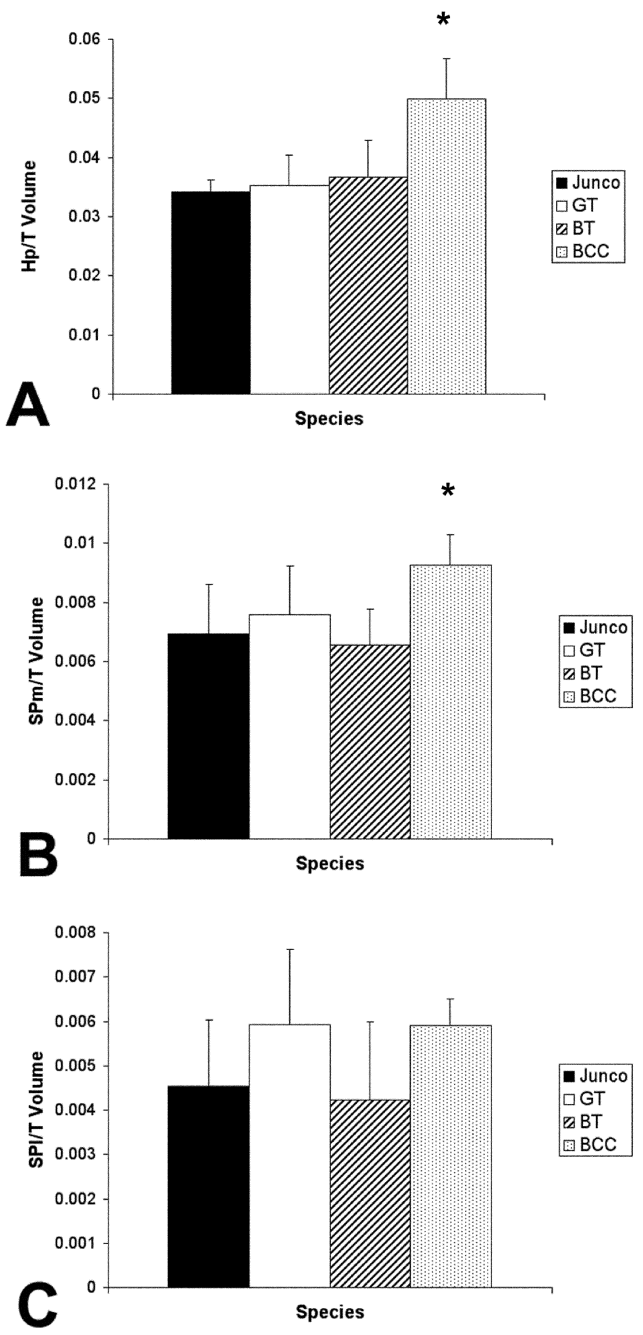

Fig. 8. (A) Mean (±S.D.) of the ratio of hippocampus over telencephalon volume for all four species. An asterisk indicates the species that was significantly different from the rest. (B) Mean (±S.D.) of the ratio of SPm over telencephalon volume for all four species. An asterisk indicates the species that was significantly different from the rest. (C) Mean (±S.D.) of the ratio of SPl over telencephalon volume for all four species.

along the lamina hyperstriatica and within lateral HA. Cells and fibers were also seen in the intercalates hyperstriatum accessorium (IHA) and hyperstriatum intercalates superior (HIS). In the mid and caudal Wulst, NPY+ fibers and cells were found along the lamina frontalis superior within the ventral HD. Along the lamina frontalis suprema, there were lightly stained NPY+ fibers within ventral HA. NPY+ cells and fibers were found throughout the rostral and caudal HA and HD, with more cells being present in the caudal part of each area. Fig. 10 illustrates the caudal level of this region in the chickadee. These findings were consistent with those in other avian species [3,4,49].

In the DVR, NPY+ beaded fibers and some NPY+ cells were found in the nucleus basalis, ectostriatum, and hyperstriatum ventrale. In the dorsal and lateral pallium, we found NPY+ cells and fibers in piriform cortex and nucleus teniae (Tn), as has been described in pigeons [1] and quail [3].

Song system nucleus MAN within the neostriatum (N) showed light NPY+ fiber staining. NPY+ fibers and cells were also found in the general region of the auditory Field L area as previously described [1]. However, we did not see NPY-ir in HVC as has been previously described in the zebra finch [16]. This could be due to real species differences or differences in the NPY antibodies or protocols used.

In the basal telencephalon, NPY-ir was found in the basal ganglia (LPO, PP, PA, TuOl, VP, Ac), nST, FPM, and the septum. There were sparse NPY+ fibers and a few small NPY+ cells in the lateral striatum (PA). The pallidum (PP) showed NPY+ fibers along with a few NPY+ cells rostrally. NPY+ fibers were also seen in the FPM. All other basal telencephalon staining was consistent with that observed in the pigeon [1] and the quail [3], including LPO, olfactory tubercle, ventral pallidum and nucleus accumbens.

The septum showed intense NPY-ir in certain areas, as it also did in quail [3]. In the dorsal aspect of the lateral septum (SLd), NPY+ fibers were very sparsely distributed, with a small band of fibers running between the Hp and septum. In the ventral aspect of the lateral septum (SLv), there were many NPY+ fibers, especially along the ventricle, which appeared to terminate on unstained cells (Fig. 11). Both the nucleus of the diagonal band (FDB) and the nucleus of the commissural septi (CoS) showed dense NPY+ fiber staining.

3.5. Diencephalon and mesencephalon

For diencephalon and mesencephalon, we emphasize any immunoreactivity that differs from that previously described, as most of the staining was similar to that found previously in other avian species. In the hypothalamus, we found staining similar to that previously described in quail [3]. In addition, we found NPY+ fibers in the stratum cellulare externum and internum. In the thalamus, there were many areas of NPY-ir, similar to that described in pigeons [4,23] and quail [3]. In addition, we found NPY+ fibers in the dorsomedial posterior thalamic nucleus (DMP), a nucleus that is part of the song system in songbirds. In the optic tectum and midbrain, NPY-ir was similar to that found in pigeons [4,21] and quail [3] with

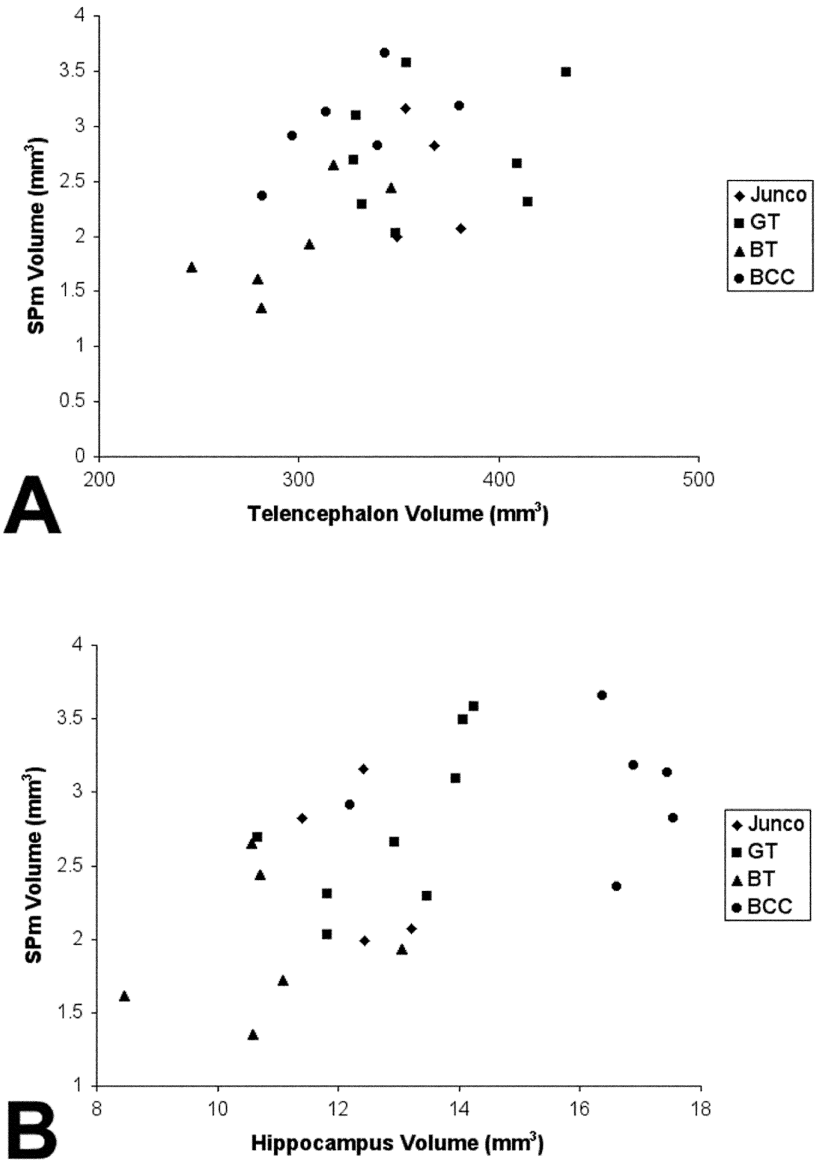

Fig. 9. (A) Scatter plot showing SPm volume plotted against hippocampal volume for all four species. (B) Scatter plot showing SPm volume plotted against telencephalon volume for all four species.

the notable exception that we saw NPY+ fibers in the substantia grisea centralis and TP as well.

3.6. Distribution of neuropeptide Y cells, fibers, and terminals in the HC

NPY-ir fibers were observed throughout the entire dorsal-ventral and rostral-caudal extent of the HC. NPY-ir cells were found in some parts of Hp in all four species (Fig. 14). In rostral sections of the Hp, they were concentrated ventrally near the midline. In middle sections, NPY+ cells were observed both ventrally and dorsomedially (Fig. 12A). In the caudal Hp, there were very few immunolabeled cells ventrally, but cells remained in the dorsomedial portion and appeared in the dorsolateral area. Many of the NPY-ir cells also showed dendritic staining (Fig. 12B).

3.7. Quantitative interspecies comparisons of neuropeptide Y

Analysis of variance revealed that there were no species differences in the number of NPY stained cells in either the ventral or dorsomedial sample areas of the Hp (ventral, $F(3,23)=1.59$, $P>0.2$; dorsomedial, $F(3,23)=1.11$, $P>0.3$) (Fig. 13). Regression analysis demonstrated a positive correlation between the number of cells in ventral and dorsomedial hippocampus ($r^2=0.70$, $F(1,26)=23.46$, $P<0.0001$) indicating that across all four species, a bird with more NPY cells in V will have more NPY cells in DM as well (Fig. 14).

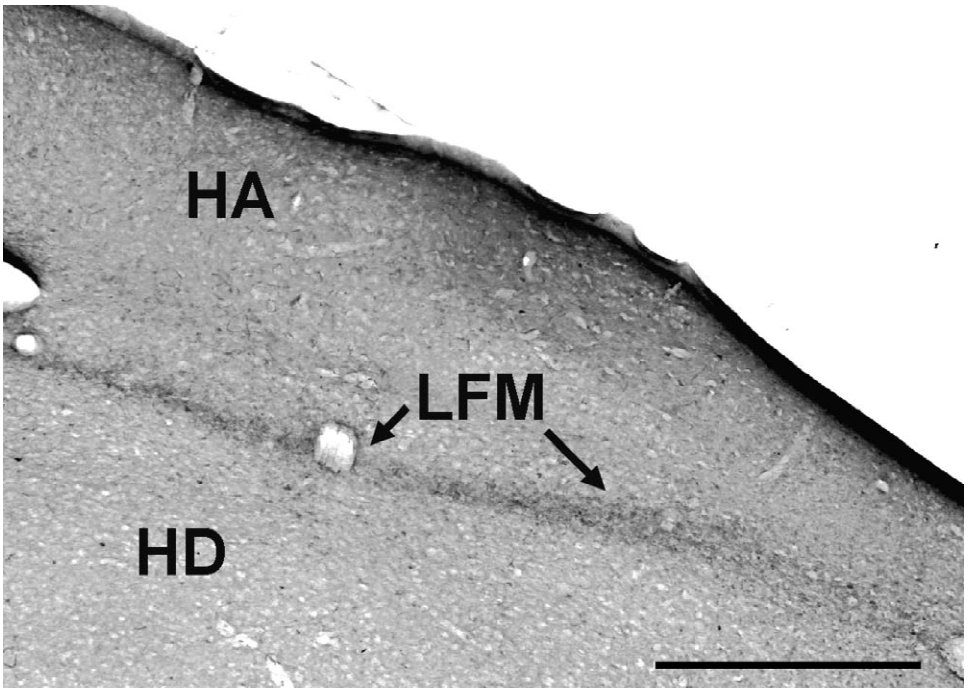

Fig. 10. A photomicrograph of a coronal section in the right hemisphere of the black-capped chickadee showing neuropeptide Y immunoreactivity in the Wulst region. HA, hyperstriatum accessorium; HD, hyperstriatum dorsale; LFM, lamina frontalis suprema. Scale bar=500 μm.

4. Discussion

4.1. Descriptive analysis of SP and NPY

Ours is the first study to describe the distribution of SP and NPY throughout the telencephalon, diencephalon, and mesencephalon of song birds. The general distribution of these peptides for the four species we examined is similar to that of SP-ir and NPY-ir in pigeons [1,4,23,42,56] and quail [3]. In both pigeons and song birds substantial labeling of SP-ir and NPY-ir fibers was observed in the visual system, particularly lateral geniculate complex, dorsal thalamic zone, and optic tectum. We also found heavily labeled SP-ir fibers in the basal ganglia, especially in fiber bundles corresponding to those in the descending tract from LPO to TP and SC in the pigeon. There were no significant differences between our observations of SP-ir fibers in songbirds and those in the pigeon [36,42,56] in these motor control areas. Similarly, our findings of NPY-ir fibers in many nuclei of the hypothalamus were consistent with previous work [3]. In mammals [9,11] and birds [43], NPY in these nuclei is thought to play an important role in the control of both feeding and reproductive behavior.

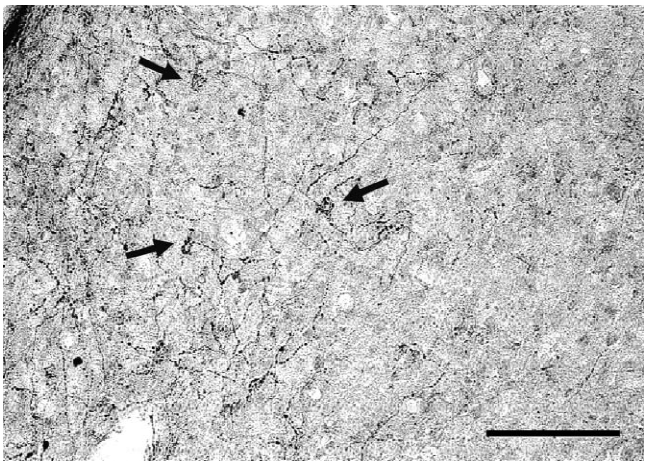

Fig. 11. A photomicrograph of a coronal section in the left hemisphere showing neuropeptide Y immunoreactivity in the septum of the dark-eyed junco. Arrows are pointing to fibers terminating on negative cells. Scale bar=200 μm.

In the septum, we found distinct patterns of NPY-ir and SP-ir, some of which have not been described in previous work. SP+ beaded fibers and cells, along with NPY+ fibers, were found in the lateral septum (SL) and nucleus of the diagonal band (FDB), with a few SP+ fibers found in the medial septum (SM). NPY+ fibers are also found along the border between hippocampus and septum. Connections between the septal area and hippocampal complex (HC) in birds [31,32,54], including reciprocal connections between HC and both SL and FDB, are similar to those in mammals [44] and it has been demonstrated that temporary inactivation of the mammalian septum affects performance on spatial memory tasks [40,41,57].

It has also been shown that the septum shows the same seasonal plasticity in volume as that found in the hippocampus for black-capped chickadees [50], and that the

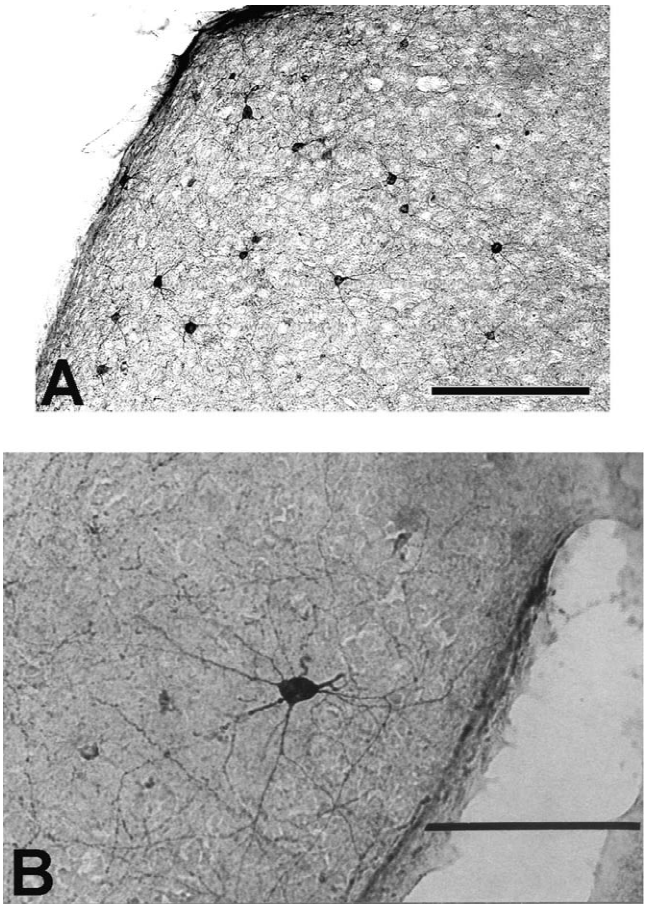

Fig. 12. (A) A photomicrograph of a coronal section in the right hemisphere showing neuropeptide Y+ cells and fibers in the dorsomedial hippocampus of the black-capped chickadee. Scale bar=200 μm. (B) A photomicrograph of a coronal section in the right hemisphere showing NPY stained cell and dendrites in the black-capped chickadee. Scale bar=100 μm.

food storing chickadee has a larger septum than two other closely related species that do not store food [48]. Further work is needed to determine whether inactivation of the avian septum affects acquisition or retrieval of spatial memory, as it does in other species.

We also found substantial NPY-ir and SP-ir in the Wulst and HC, which are the areas of focus in this study. The rostral-most portion of the Wulst is thought to carry out functions of mammalian primary somatosensory and motor cortices, while the mid to caudal portion is thought to carry out the functions of primary visual cortex [37]. The adjacent HC, comprised of the APH and Hp, is known to be important in spatial memory [12,19,46]. In the species studied here, intense SP-ir was observed within the Wulst and APH. Although the overall distribution of SP-ir in the Wulst and APH of our four song bird species is highly consistent with data reported from pigeons [4,14,49], we found two distinct SP areas within these regions which we designated SPm and SPl. SPm was found at the boundary of the Hp within the APH and SPl was found within the mid to caudal portions of the hyperstriatum accessorium (HA) of the Wulst.

SPm appears to correspond to area 7 in Ref. [14] and the SP field (SPf) in Ref. [53]. In Ref. [49], the SP immunoreactivity described in the dorsal APH also corresponds to SPm in our tissue. SPl appears to correspond to the SP-ir described within the ventral HA in [4]. The staining in ventral HA and hyperstriatum dorsale shell (HD-shell) in Ref. [49] corresponds roughly to SPl as well. Although these areas of fiber and terminal labeling corresponded to those found in pigeons [4,49], our tissue did not show SP-like immunoreactive cells in these areas as it did in pigeons.

In the Hp proper of the juncos, we found a distinct group of SP+ cells along the ventricle and many SP+ fibers distributed throughout the entire Hp. This appears to be similar to observations in pigeons [4,14,49]. However, these cells and fibers were not present in the three tit species. Perhaps this is a family difference. Juncos are part of the Passeridae family. It would be useful to look at other species from this family to see if the same SP-ir in the Hp is present. Another explanation could be differences in natural history and behavior. All three parid species used in this study are non-migratory and the juncos are migratory. This could be studied by examining other migratory species, by comparing non-migratory populations of juncos to those that migrate, as well as by comparing juncos during non-migration and migration periods. A neurochemical explanation could be that the juncos are sequestering substantially more SP in their cell bodies than the other species. The parids may have cells in the Hp that produce SP, but are transporting the neuropeptide out of the cell body very quickly. This hypothesis could be tested in tissue from birds that have been treated with an axonal transport inhibitor.

4.2. Quantitative analyses of SP

This study replicates the finding that a food-storing bird in the parid family has a larger relative hippocampal volume than representative non-storing species both within and outside the same family [24,26,33,45]. Across the four species of birds, the volume of SPm is correlated with hippocampus size. In other words, birds with a larger relative hippocampal volume tend to have a larger relative SPm area. Indeed, neurons in the SP field (SPf) project to the dorsolateral Hp as determined with PHAL tract tracing [53,54] and Golgi study [38]. In addition, there are projections from Hp back to SPm and nearby areas [10].

The results also demonstrate that black-capped chickadees have a larger SPm area relative to telencephalon than the other three species. We have not shown a direct association between spatial memory behavior and the SPm region. However, substance P release may be a memory modulator as injection of SP peripherally or directly into nucleus basalis magnocellularis has produced memory-

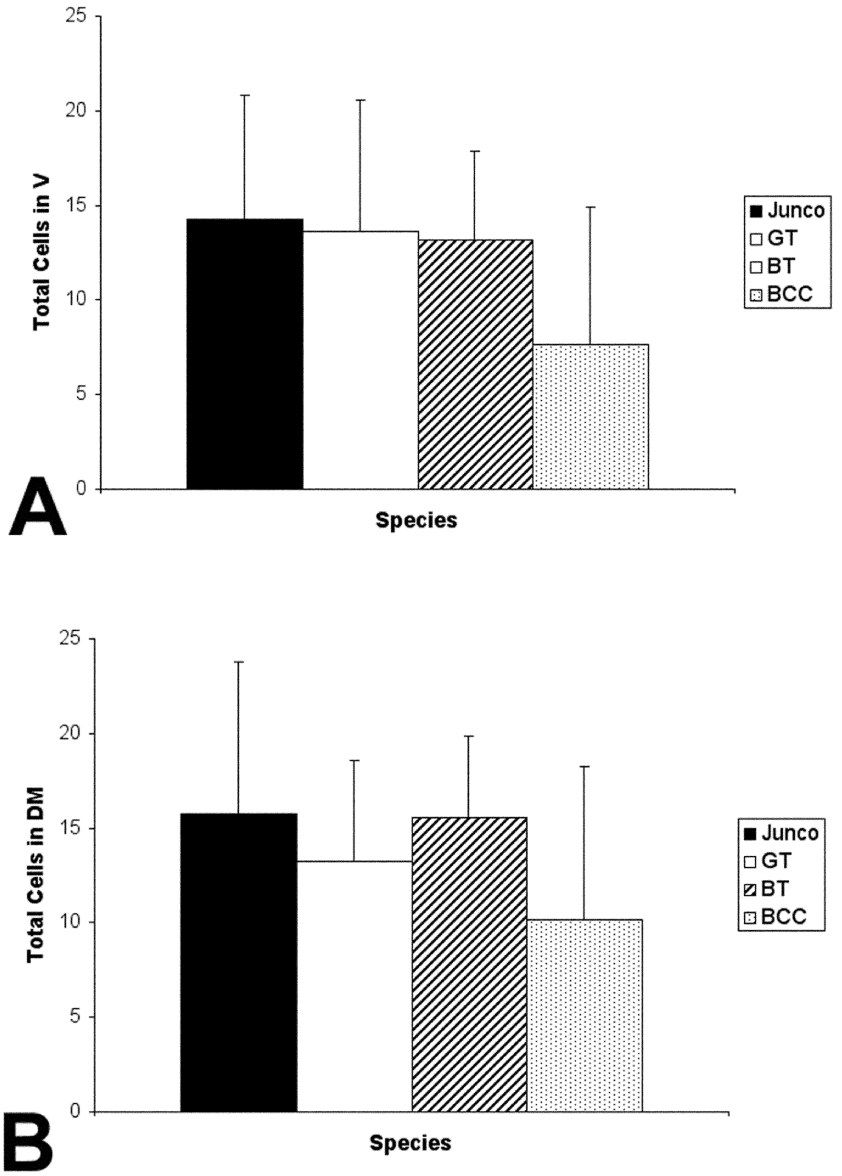

Fig. 13. (A) Mean (±S.D.) of the total number of cells in the ventral hippocampus for all four species. (B) Mean (±S.D.) of the total number of cells in the dorsomedial hippocampus for all four species.

promoting effects in a variety of learning tasks in mammals and fish [27,35,55]. We find that SP is present in a brain region connected to the hippocampus. Thus, this region could participate with the hippocampus in forming spatial memories, a hypothesis that could be assessed by investigating its morphology and function in other food storing birds in the parid family.

4.3. Quantitative analyses of NPY

There were no significant species differences in the number of NPY cells found in the ventral and dorsomedial area of the Hp. However, a positive correlation between the number of NPY-ir cells in these two areas was found in all four species. Birds with a higher number of NPY cells in one area also had a higher number of cells in the other. This suggests a link between ventral and dorsomedial hippocampus in the distribution of NPY cells, which could be related to the intrinsic anatomical connectivity between these two areas [54].

The connection between NPY production and behavioral function is not clear. NPY stimulates food intake in both mammals [11] and birds [43] when it is injected into specific hypothalamic nuclei. Injection of NPY into the rostral mouse hippocampus improves memory retention for training in a T-maze [18]. There is also a very high concentration of NPY cell bodies in the mammalian hippocampus [22]. In birds, nerve growth factor (NGF) enhances NPY immunoreactivity in many areas of the brain, while anti-NGF decreases the number of NPY cells

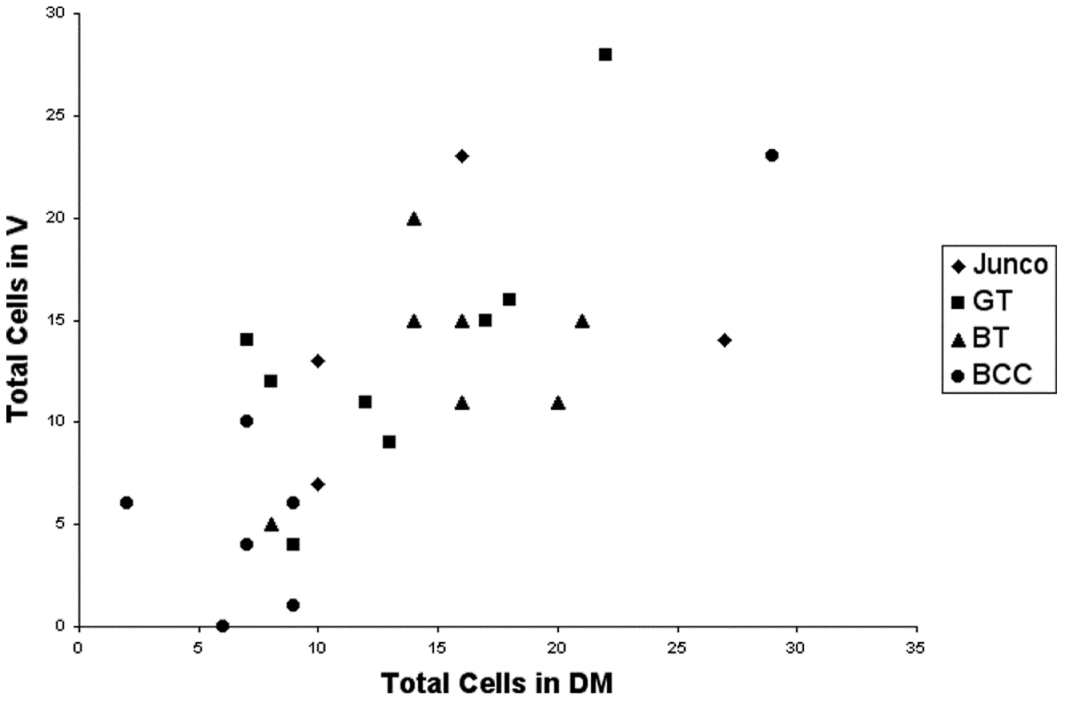

Fig. 14. Scatter plot showing the total number of NPY+ cells in the ventral hippocampus plotted against the total number of NPY cells in the dorsomedial hippocampus for all four species.

in the Hp [16]. NGF aids in maintaining the survival of new neurons and connections, so may be important in certain types of plastic learning and memory. Double-label studies using pigeons find that a large percentage of telencephalic neurons that contain NPY also contain somatostatin, which is similar to the co-localization of these two neuropeptides in mammals [1]. These two neuropeptides may be acting together to regulate certain telencephalic functions.

If NPY does facilitate memory and stimulate food intake in the avian brain, one might expect to find higher production of NPY in certain areas of the hippocampus during the time when the bird is storing more food and therefore forming many new spatial memories. This could be assessed by measuring the distribution and number of NPY cells in birds captured during the peak of food-storing in the fall and compared to those captured in spring. Injection of NPY into the avian hippocampus during spatial tasks in the laboratory would also be of interest. Since NPY plays a role in regulating food intake, it is possible that variation in the expression of this peptide could also be linked to the onset and regulation of food-storing.

5. Conclusions

We find no species differences in the number of NPY cells in the ventral or dorsomedial avian hippocampus. Further studies will be needed to determine whether this peptide modulates hippocampal function. The overall distribution of NPY cells and fibers in the four songbird species of this study is consistent with that found in pigeons, demonstrating conservative distribution of NPY within the avian brain.

The two SP areas we found in the current study, SPm and SPl, are conserved, both in their neuroanatomical distribution within the avian brain and in the large quantity of SP that is reliably observed in these areas. We provide the first evidence of significant species differences in the relative volume of one of these areas (SPm) and show a positive correlation between Hp and SPm volumes. The function of the avian SPm area remains to be determined.

The significance of the species differences in SPm volume also remains to be determined. Future work will determine whether this region is involved in forming spatial memories and whether it participates in the seasonal plasticity shown by the hippocampus.

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